AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Virology®

VACCINES AND ANTIVIRAL AGENTS

T Cell Responses Induced by Attenuated Flavivirus Vaccination Are Specific and Show Limited Cross-Reactivity with Other Flavivirus Species

Alba Grifoni,[a] Hannah Voic,[a] Sandeep Kumar Dhanda,[a] Conner K. Kidd,[a] James D. Brien,[b] Søren Buus,[c] Anette Stryhn,[c] Anna P. Durbin,[d] Stephen Whitehead,[e] Sean A. Diehl,[f] Aruna D. De Silva,[a,g] Angel Balmaseda,[h] Eva Harris,[i] Daniela Weiskopf,[a] Alessandro Sette[a,j]

[a]Division of Vaccine Discovery, La Jolla Institute for Immunology, La Jolla, California, USA
[b]Saint Louis University, Saint Louis, Missouri, USA
[c]Laboratory of Experimental Immunology, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark
[d]Johns Hopkins University Bloomberg School of Public Health, Baltimore, Maryland, USA
[e]National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA
[f]University of Vermont School of Medicine, Burlington, Vermont, USA
[g]Department of Paraclinical Sciences, General Sir John Kotelawala Defense University, Ratmalana, Sri Lanka
[h]National Virology Laboratory, National Center for Diagnosis and Reference, Ministry of Health, Managua, Nicaragua
[i]Division of Infectious Diseases and Vaccinology, School of Public Health, University of California, Berkeley, California, USA
[j]Department of Medicine, University of California San Diego, La Jolla, California, USA

ABSTRACT Members of the flavivirus genus share a high level of sequence similarity and often circulate in the same geographical regions. However, whether T cells induced by one viral species cross-react with other related flaviviruses has not been globally addressed. In this study, we tested pools of epitopes derived from dengue (DENV), Zika (ZIKV), Japanese encephalitis (JEV), West Nile (WNV), and yellow fever (YFV) viruses by intracellular cytokine staining (ICS) using peripheral blood mononuclear cells (PBMCs) of individuals naturally exposed to DENV or immunized with DENV (TV005) or YF17D vaccine. CD8 T cell responses recognized epitopes from multiple flaviviruses; however, the magnitude of cross-reactive responses was consistently severalfold lower than those to the autologous epitope pools and was associated with lower expression of activation markers such as CD40L, CD69, and CD137. Next, we characterized the antigen sensitivity of short-term T cell lines (TCL) representing 29 different individual epitope/donor combinations. TCL derived from DENV monovalent vaccinees induced CD8 and CD4 T cells that cross-reacted within the DENV serocomplex but were consistently associated with >100-fold-lower antigen sensitivity for most other flaviviruses, with no cross-recognition of YFV-derived peptides. CD8 and CD4 TCL from YF17D vaccinees were associated with very limited cross-reactivity with any other flaviviruses and in five out of eight cases >1,000-fold-lower antigen sensitivity. Overall, our data suggest limited cross-reactivity for both CD4 and CD8 T cell responses between flaviviruses and have implications for understanding immunity elicited by natural infection and strategies to develop live attenuated vaccines against flaviviral species.

IMPORTANCE The envelope (E) protein is the dominant target of neutralizing antibodies for dengue virus (DENV) and yellow fever virus (YFV). Accordingly, several DENV vaccine constructs use the E protein in a live attenuated vaccine format, utilizing a backbone derived from a heterologous flavivirus (such as YF) as a delivery vector. This backbone comprises the nonstructural (NS) and capsid (C) antigens, which are dominant targets of T cell responses. Here, we demonstrate that cross-reactivity at the level of T cell responses among different flaviviruses is very limited, despite

Citation Grifoni A, Voic H, Dhanda SK, Kidd CK, Brien JD, Buus S, Stryhn A, Durbin AP, Whitehead S, Diehl SA, De Silva AD, Balmaseda A, Harris E, Weiskopf D, Sette A. 2020. T cell responses induced by attenuated flavivirus vaccination are specific and show limited cross-reactivity with other flavivirus species. J Virol 94:e00089-20. https://doi.org/10.1128/JVI.00089-20.

Editor Susana López, Instituto de Biotecnologia/UNAM

Address correspondence to Alessandro Sette, alex@lji.org.

Received 17 January 2020
Accepted 25 February 2020
Accepted manuscript posted online 4 March 2020
Published 4 May 2020

high levels of sequence homology. Thus, the use of heterologous flavivirus species as a live attenuated vaccine vector is not likely to generate optimal T cell responses and might thus impair vaccine performance.

KEYWORDS DENV, T cells, YFV, flaviviruses, vaccines

Flavivirus infections can cause a wide variety of clinical manifestations and complications in humans, ranging from undifferentiated fever, vascular leak syndrome, encephalitis, and death. Because of their high prevalence worldwide, the four serotypes of dengue virus (DENV), yellow fever virus (YFV), West Nile virus (WNV), Japanese encephalitis virus (JEV), and, most recently, Zika virus (ZIKV) are responsible for tens of millions of disease cases and thus have a large global impact on human health and disease (1, 2).

Despite intense investigation, the immune mechanisms behind disease and vaccine efficacy are not well defined, particularly in the case of DENV and ZIKV (3). Both antibody and T cell responses have been reported to play a role in immunity and immunopathology (4–10). Of particular interest in this context are the potential contribution of flavivirus cross-reactive antibodies and T cell responses to both disease protection and immunopathogenesis.

The envelope (E) protein, a major virion surface protein, is involved in receptor binding and membrane fusion and induces neutralizing antibodies in infected hosts. Human infection results in the production of both virus species-specific and flavivirus cross-reactive antibodies (11). In the case of DENV, most individuals generate cross-reactive antibodies that initially protect against the spread of infection but may later enhance infection and/or disease with heterologous serotypes (3, 5). Similarly, cross-neutralization in acute ZIKV infection in donors with preexisting DENV immunity was strongest in early convalescence but waned to low levels over time (12). It has been shown in *in vitro* assays that JEV vaccination induced high levels of JEV neutralizing antibodies but also DENV cross-reactive antibodies, which at subneutralizing levels possessed DENV infection enhancement activity (13).

At the level of T cell reactivity, a similar pattern has been reported, with clear cross-reactivity within different DENV serotypes. It has been proposed that cross-reactive T cells raised against the original infecting serotype dominate during a secondary heterologous infection, a phenomenon that has been termed "original antigenic sin" (14, 15). It was hypothesized that during secondary infection, expansion of preexisting, lower avidity, and cross-reactive memory T cells may induce a "cytokine storm" contributing to immunopathogenesis (15). In contrast with this initial theory, several lines of evidence suggest that both CD4 and CD8 T cells are involved in resolving DENV infection. It has been demonstrated that both CD4 and CD8 T cells can have a direct role in protection against DENV challenge in a murine model (16, 17), and strong, multifunctional T cell responses correlated with alleles associated with protection from severe disease in humans naturally exposed to DENV (10, 18–21). These data implied a protective role for T cells against severe DENV disease (9). Similarly, it has been demonstrated that T cell immunity to ZIKV and DENV induced responses that are cross-reactive with other flaviviruses in both humans (22) and HLA transgenic mice (23).

Vaccines for JEV and YFV, but not for WNV or ZIKV (24), are currently licensed for use in humans and are based on live attenuated vaccine (LAV) or inactivated platforms. A DENV LAV, based on a chimeric DENV/YFV, was recently licensed, but significant controversy remains over its safety and efficacy (25, 26). While all licensed vaccines rely on serological markers as immune correlates measured with validated assays (3), the potential role of T cell-mediated immunity is not yet fully understood. This is relevant since a general hallmark of LAVs is their ability to induce both humoral and cellular immune memory. We previously defined in the DENV context the antigens recognized as immunodominant by both CD8 and CD4 responses (9, 27–29). Nonstructural (NS) proteins NS3, NS4B, and NS5 were the dominant antigens for CD8 T cell responses,

TABLE 1 Source proteins of peptides contained in the flavivirus CD8 MPs

Virus	No. of peptides from each viral protein category			
	prM and envelope	Capsid	Nonstructural	Total
DENV	49	14	205	268
ZIKV	52	10	247	309
YFV	70	11	287	368
JEV	50	10	250	310
WNV	60	13	251	324

while for CD4 T cell responses, the capsid (C), together with NS2A, NS3, and NS5, was immunodominant (28).

Over the past few years, several full-length live attenuated vaccines containing antigens from all four DENV serotypes (tetravalent vaccines) have been developed. The National Institute of Allergy and Infectious Diseases has developed the live attenuated dengue vaccines TV003 and TV005 containing attenuated DENV1, DENV3, and DENV4 plus a chimeric DENV2/4 virus (30), while Takeda's live attenuated tetravalent dengue vaccine candidate (TAK-3) is comprised of an attenuated DENV2 strain plus chimeric viruses containing the prM and E genes of DENV1, -3, and -4 cloned into the attenuated DENV2 backbone (31). Thus, both vaccines would be expected to elicit cellular immunity cross-reactive among different serotypes. In fact, T cell responses following tetravalent vaccination with TV005 are focused on the highly conserved NS proteins (32). Likewise, it has been reported that TAK-003, which is based on a DENV2 NS backbone, induces significant cross-reactive responses against NS proteins of DENV1, -3, and -4 (33).

The most advanced vaccine against dengue virus, Dengvaxia, is based on chimeric viruses containing the prM and E genes of DENV1, -2, -3, and -4 cloned into the attenuated YF backbone (34). This vaccine has been associated with low efficacy as well as safety issues (26). In the case of the Dengvaxia vaccine, CD8 cellular immunity will have to rely on YF/DENV T cell cross-reactivity, since the NS proteins encoded in the vaccine are derived from YFV and not DENV. A potential decreased or compromised cellular immunity might be a potential factor contributing to the lower efficacy. Thus, it is of interest to address to what extent DENV and YFV responses induced by vaccination are cross-reactive.

Here, we characterize immune responses elicited by the TV005 and YF17D vaccines to identify and define the functional attributes of cross-reactive responses at the single-epitope level between different flaviviruses. The majority of TV005-induced CD4 and CD8 T cells recognize the DENV serocomplex, while the YF17D vaccine induced fewer cross-reactive T cells. Characterization of the extent and functionality of CD4 and CD8 T cell cross-reaction across different flaviviruses will contribute to the understanding of immunity in natural infections and has particular implications for vaccine efficacy and safety in settings of endemicity.

RESULTS

Sequence homology of CD8 epitope pools representative of five prevalent flavivirus species. To address to what extent T cells induced by live attenuated DENV or YF vaccines cross-react with other flaviviruses, we developed pools of several hundred predicted or experimentally defined CD8 epitopes from five prevalent flaviviruses (DENV, ZIKV, YFV, WNV, and JEV). The process used to define each of these epitope megapools (MPs; composed of 9-mers and 10-mers) is described in more detail in Materials and Methods. As shown in Table 1, each MP contained an average of 316 peptides (ranging from 268 to 368 peptides/pool) derived from all 10 proteins (C, M/E, and NS1 to -5).

Table 2 lists the percentage of peptides for each MP that shared 70% or more sequence identity with DENV, ZIKV, YFV, WNV, and JEV consensus sequences (35). As expected, based on various degrees of homology between the different viruses, the number of conserved epitopes was highest between DENV and ZIKV and between WNV

TABLE 2 Sequence identities of CD8 flavivirus MPs and consensus sequences of indicated flaviviruses[a]

Virus	% of peptides with ≥70% sequence identity to consensus sequence							
	DENV	ZIKV	YFV	JEV	WNV	CHIKV	EBOV	HCV
DENV	83	33	16	25	26	0	0	0
ZIKV	30	100	16	35	33	0	0	0
YFV	17	16	100	15	17	0	0	0
JEV	26	30	16	100	69	0	0	0
WNV	28	33	16	71	100	0	0	0

[a]The percent sequence identity in each DENV serotype (DENV1, -2, -3, and -4) was calculated independently and the maximum value was assigned to represent the DENV sequence identity.

and JEV. None of the epitopes included in the various MPs shared 70% or more sequence identity with control viral sequences derived from the Ebola virus (EBOV), Chikungunya virus (CHIKV), and hepatitis C virus (HCV).

Measuring CD8 T cell responses in areas where flavivirus is endemic. Addressing the extent of T cell cross-reactivity among several flaviviruses is important to understand the potential impact of exposure to multiple subsequent flaviviruses in areas where flavivirus is endemic. To address this point, our overall approach was to assess the ability of heterologous flavivirus MPs to elicit the production of gamma interferon (IFN-γ) from memory $CD8^+$ T cell responses in samples from Nicaragua and Sri Lanka. To determine whether DENV-specific T cell responses might be cross-reactive with other flavivirus epitopes, we studied peripheral blood mononuclear cell (PBMC) samples from blood bank donors in Managua, Nicaragua ($n = 8$), and Colombo, Sri Lanka ($n = 6$), previously selected to be DENV seropositive and categorized as high responders against the DENV MP (the high responders were defined by a stimulation index of >2 and background reactivity below 0.1; see Materials and Methods). Figure 1 shows the reactivity against all five MPs expressed as a percentage of $CD3^+$ $CD8^+$ IFN-γ-producing cells. As expected due to the selection criteria of those donors, significantly high reactivity was observed after DENV MP stimulation, with a geometric mean response of 0.24 ($P = 0.0001$ compared to the same unstimulated cells as a control [CTRL] with paired nonparametric Wilcoxon test). In addition, significant reactivity in DENV MP-reactive donors to ZIKV, YFV, WNV, and JEV MPs was observed, with

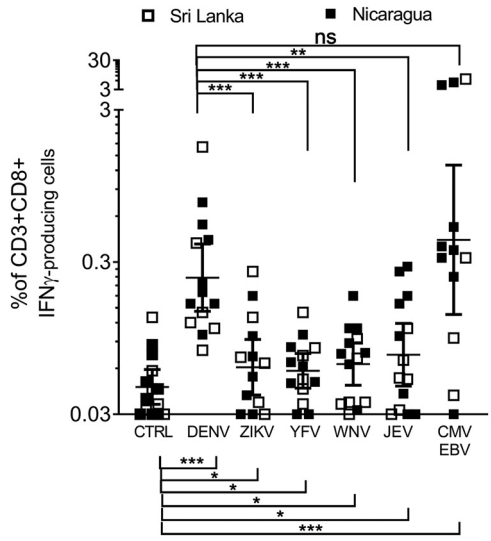


FIG 1 CD8 reactivity against flavivirus MP in areas where flavivirus is endemic. Shown are percentages of $CD3^+$ $CD8^+$ IFN-γ-producing T cells after flavivirus MP stimulation for 6 h of PBMCs derived from blood bank donors in Nicaragua ($n = 8$) and Sri Lanka ($n = 6$). Statistical analyses were performed using paired nonparametric Wilcoxon test. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. ns, not significant.

geometric mean in the range of 0.056 to 0.074 (*P* values compared to the control were 0.048 for ZIKV, 0.013 for YFV, 0.037 for WNV, and 0.046 for JEV). These results demonstrated that five heterologous MPs recalled a significant response in DENV MP-reactive subjects compared to an unstimulated control.

To determine the extent of cross-reactivity, we next compared the magnitude of the homologous responses elicited by the DENV MP in DENV-reactive donors with the heterologous responses elicited by the ZIKV, YFV, JEV, and WNV MPs and found that the heterologous MP responses were significantly lower in magnitude than the homologous MP responses (*P* values ranging from 0.0005 to 0.0479).

Additionally, we also assessed T cell reactivity in this cohort against a nonflavivirus cytomegalovirus/Epstein-Barr virus (CMV/EBV) MP. We found a significant T cell response when comparing the CMV/EBV MP to the unstimulated control (geometric mean of the response of 0.42 and *P* value of 0.0005) but no significant difference in terms of T cell reactivity compared to the DENV MP ($P = 0.4697$).

These results are compatible with the notion that DENV-reactive CD8 T cells responses might recognize certain cross-reactive epitopes contained in the other MPs, although to a significantly lower extent in terms of magnitude of response, as the geometric mean percentage of $CD3^+$ $CD8^+$ IFN-γ^+ cells in each heterologous flavivirus MP is 4- to 5-fold less than observed after DENV MP stimulation. As some of these samples were collected in Sri Lanka, an area of endemicity where other flaviviruses are circulating, it cannot be excluded that some of the response detected was due to exposure to the other flaviviruses. Whether this was indeed the case could not be addressed in the Sri Lanka samples, as they were derived from buffy coats from normal blood donations and thus neither clinical history details nor serum samples were available. In Nicaragua, however, the PBMCs were collected before the introduction of ZIKV, and no YFV, WNV, or JEV is known to be circulating; additionally, there is no YFV vaccination of the general public. Thus, previous exposure to other flaviviruses is highly unlikely in the Nicaraguan samples.

Cross-reactivity pattern of CD8 T cell responses induced by a tetravalent dengue vaccine (TV005). To address potential cross-reactive responses in a controlled exposure setting, and exclude the possibility that previous unknown flavivirus exposure influences the results obtained, we utilized a cohort of US donors who were vaccinated with experimental tetravalent dengue live attenuated vaccine (TDLAV) candidate TV005 6 to 12 months prior to blood collection. All of these donors were confirmed to be flavivirus naive before vaccine administration (36).

Specifically, we tested the CD8 T cell IFN-γ reactivity against all five flavivirus pools (DENV, ZIKV, YFV, WNV, and JEV) in PBMCs derived from TDLAV-vaccinated and unvaccinated flavivirus-naive control donors (Fig. 2A). As expected, no reactivity to either MP was observed in the case of the unvaccinated controls (no significant differences between the DENV MP and CTRL groups). Also as expected, the strongest reactivity was detected in the case of TDLAV vaccinees against the DENV MP, with a geometric mean response of CD8 T cells producing IFN-γ of 0.22 ($P = 0.0001$ compared to the unstimulated control by paired nonparametric Wilcoxon test, and $P < 0.0001$ compared to the DENV MP-stimulated unvaccinated group by unpaired nonparametric Mann-Whitney test).

Statistically significant but weak responses in TDLAV vaccinee samples were detected against ZIKV, YFV, and JEV MPs, with geometric mean values in the range of 0.045 to 0.063 (*P* values compared to either the CTRL or the flavivirus-naive donors were 0.0007 and 0.0088, respectively, for ZIKV, 0.0014 and 0.004 for YFV, and 0.0002 and 0.005 for JEV).

In the case of the WNV MP, the responses were not significantly higher than the control ($P = 0.058$) and were significantly lower than the DENV MP ($P = 0.016$). Finally, the nonflavivirus MP (CMV/EBV) responses were higher than the control but, as expected, did not differ between the vaccinated and unvaccinated groups ($P = 0.0008$ and 0.81). Based on these results, we conclude that TDLAV vaccination induced CD8

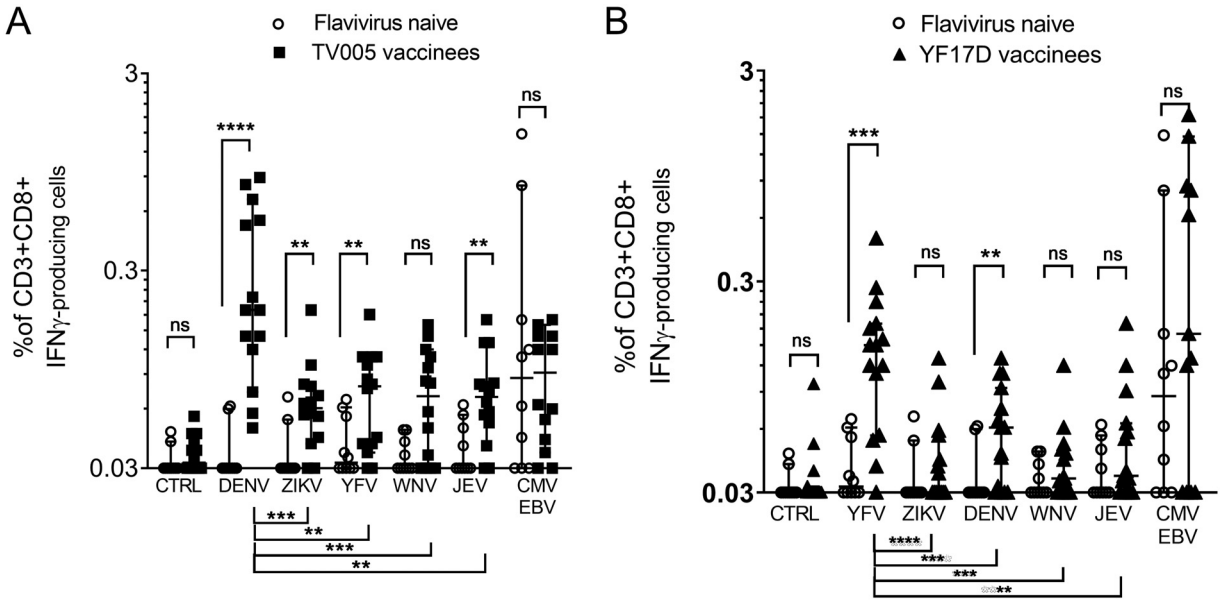


FIG 2 CD8 reactivity against flavivirus MP in vaccination. Shown are percentages of $CD3^+$ $CD8^+$ IFN-γ-producing T cells after flavivirus MP stimulation for 6 h. (A) Reactivity of TV005 vaccinees ($n = 14$) compared to that of flavivirus-naive subjects. ($n = 10$). (B) Reactivity of YF17D vaccinees ($n = 15$) compared to the same flavivirus-naive cohort. Data are expressed as geometric means with 95% confidence intervals (CI). Statistical analyses between the different cohorts were performed using unpaired nonparametric Mann-Whitney test, while statistical analyses for the same cohort across stimuli were performed using paired nonparametric Wilcoxon test. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

responses that are also capable of recognizing ZIKV, YFV, and JEV epitopes and that the results were indeed flavivirus specific.

We next examined the level of cross-reactive responses in terms of magnitude. In all cases, cross-reactive responses in TDLAV vaccinee samples were significantly lower (4.6-fold lower on average than with DENV MP stimulation; $P = 0.0004$, 0.0012, 0.002, and 0.0001 for the ZIKV, YFV, JEV, and WNV MPs, respectively). Thus, we conclude that TDLAV vaccination induces CD8 responses that are also capable of recognizing ZIKV, YFV, and JEV epitopes but to a significantly lower extent (Fig. 2A).

Flavivirus cross-reactive CD8 T cell responses induced by the yellow fever vaccine (YF17D). We next asked whether a similar pattern of cross-reactivity might be detectable after vaccination with a different attenuated flavivirus vaccine. Accordingly, we tested the CD8 T IFN-γ reactivity against all five flavivirus pools in PBMCs isolated from U.S. donors 6 to 12 months after vaccination with the live attenuated yellow fever vaccine (YFLAV; YF17D) and from unvaccinated U.S. controls (Fig. 2B).

As expected, little to no reactivity to the YFV MP was observed in the case of the unvaccinated controls (no difference between YFV MP and CTRL groups). In contrast, and also as expected, the strongest reactivity was detected against the YFV MP, with a geometric mean of 0.122 ($P < 0.0001$ compared to the unstimulated control and $P = 0.0002$ compared to the YFV MP-stimulated unvaccinated group).

Responses were noted also in the case of the YFLAV vaccinees (compared to either the control or the flavivirus-naive donors) when stimulated with the DENV MP ($P = 0.0003$ and 0.0016, respectively). Some responses were also noted in the case of the ZIKV, WNV, and JEV MPs, with median values in the range of 0.027 to 0.049. These responses were, in general, significant compared to the CTRL but not significant compared to unvaccinated donors (ZIKV, $P = 0.0461$ and 0.3446; WNV, $P = 0.0001$ and 0.3168; and JEV, $P < 0.0001$ and $P = 0.1473$, respectively). Finally, as previously stated, no significant difference between YFLAV vaccinees and flavivirus-naive controls was observed in the case of a control MP encompassing epitopes derived from the nonflaviviruses CMV and EBV ($P = 0.0046$ and 0.3352). Based on these results, we conclude that YFLAV vaccination induces CD8 responses that are capable of recognizing DENV epitopes but ZIKV, WNV, and JEV epitopes only marginally, if at all.

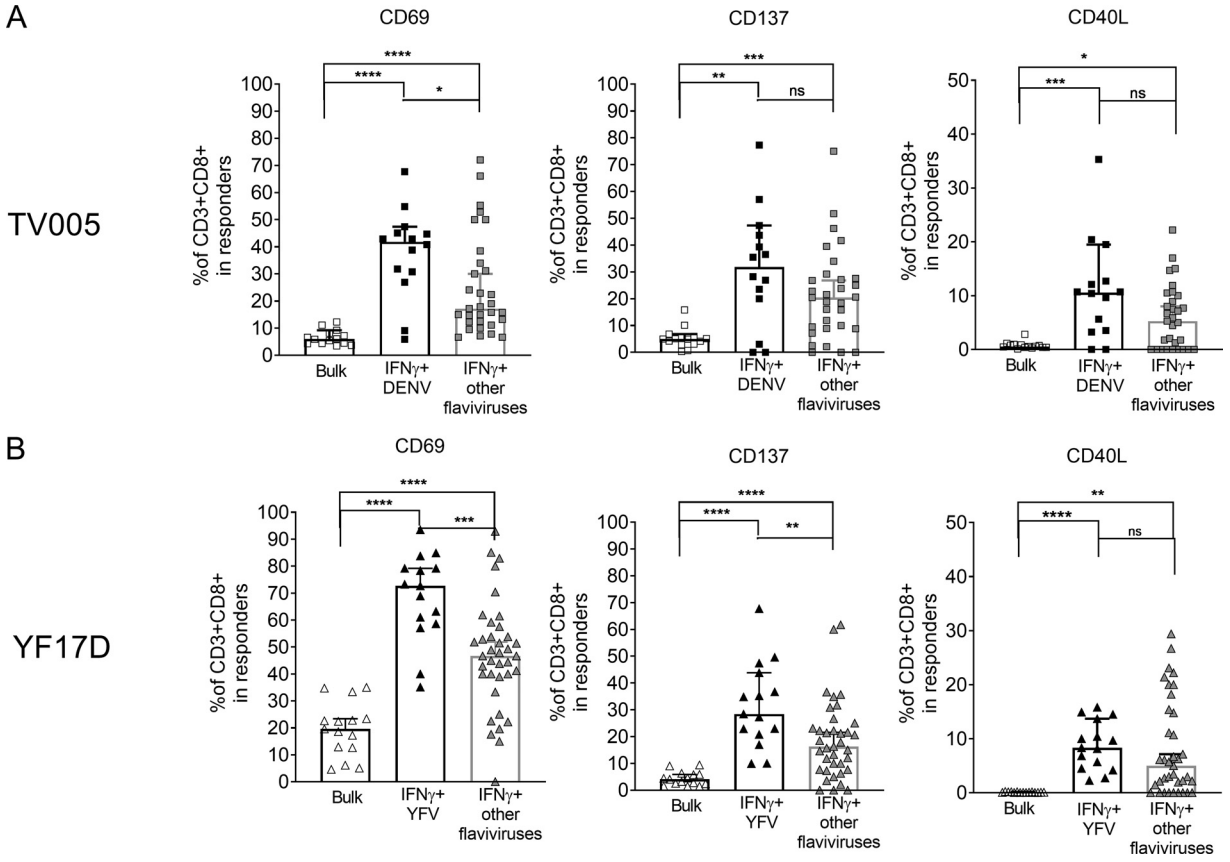


FIG 3 Activation marker expression in IFN-γ-producing CD8 T cells after flavivirus-specific MP stimulation or cross-reactive flavivirus MP stimulation. Expression of CD69, CD137, and CD40L (for gating strategy, see Fig. 8) was assessed for individual donor/MP stimulation combinations. Only instances associated with positive responses were examined (defined as percentage of $CD3^+$ $CD8^+$ IFN-γ^+ cells above the 0.03 threshold calculated based on the mean + 2 SDs of flavivirus-naive MP reactivity). Expression of these markers in the $CD3^+$ $CD8^+$ IFN-γ^+ subset is compared with bulk $CD3^+$ $CD8^+$ T cells (white symbols) after stimulation with homologous MP (black symbols) or heterologous MPs (all different heterologous MPs combined; gray symbols). Responses in TV005 vaccinees (A; squares, $n = 14$) and YF17D vaccinees (B; triangles, $n = 15$) are shown. Data are expressed as medians with 95% CI. Statistical analyses were performed using unpaired nonparametric Mann-Whitney test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

We next compared the magnitude of the YFV MP responses in YFLAV recipients with the responses to the DENV, ZIKV, WNV, and JEV MPs. In all cases, responses against DENV, ZIKV, WNV, and JEV MPs were significantly lower (3.4-fold lower, on average, than to YFV MP; $P < 0.0001$, 0.0006, 0.0001, and 0.0012 for the ZIKV, DENV, WNV, and JEV MPs, respectively). We conclude that YFLAV vaccination is able to induce cross-reactive CD8 T cell responses recognizing epitopes derived from other flaviviruses, but the magnitude of such cross-reactive responses is significantly lower than for homologous YFV-derived epitopes (Fig. 2B).

CD8 T cell cross-recognition of heterologous epitopes is associated with lower expression of activation markers. The results above suggest that heterologous cross-reactive responses are in general weaker than the homologous induced responses when stimulated with peptide variants. We next investigated whether, in addition to the difference in magnitude, we could observe differences in the quality of CD8-specific T cell responses, as represented by activation markers. For this purpose, we analyzed the intracellular expression of CD40L, CD69, and CD137 activation markers in virus-specific CD8 T cells ($CD3^+$ $CD8^+$ IFN-γ^+) after stimulation with the various MPs (see Fig. 8 for gating strategy). The percentages of total CD8 T cells expressing the CD40L, CD69, and CD137 markers (Fig. 3, white symbols) were compared with the percent expression in $CD3^+$ $CD8^+$ IFN-γ^+ T cells after DENV CD8 MP stimulation (Fig. 3, black symbols) or after stimulation with other flavivirus MPs (Fig. 3, gray symbols).

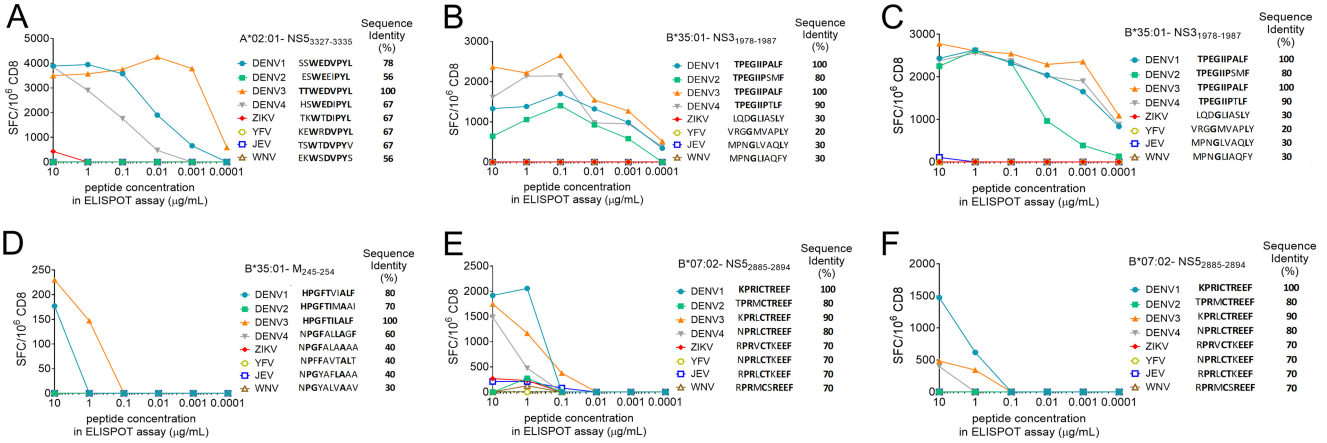
For TDLAV (TV005) vaccinees, CD69, CD137, and CD40L markers were significantly upregulated in the T cells responding to DENV MP stimulation compared to the bulk population (CD69, DENV [median = 42%] versus bulk [6%], $P < 0.0001$; CD137, DENV [32%] versus bulk [5%], $P = 0.0057$; and CD40L, DENV [11%] versus bulk [0.6%], $P = 0.0008$; Mann-Whitney test). We then compared the expressions of these markers on $CD3^+$ $CD8^+$ IFN-γ^+ T cells from TDLAV (TV005) vaccinees in response to DENV-specific and heterologous MP stimulation. A significant lower expression of CD69 was detected after stimulation with DENV MP versus other flavivirus MPs ($P = 0.0344$), and a nonsignificant trend was observed for CD40L (DENV-specific versus other flavivirus MPs, $P = 0.0575$) and for CD137 (DENV-specific versus other flavivirus MPs, $P = 0.1033$) (Fig. 3A).

Similarly, YFV-specific homologous stimulation of YFLAV (YF17D) vaccinees was associated with significantly increased expression of CD69, CD137, and CD40L markers compared to the bulk population (CD69, YFV [73%] versus bulk [0.1%], $P < 0.0001$; CD137, YFV [28%] versus bulk [4%], $P < 0.0001$; and CD40L, YFV [8%] versus bulk [0.1%], $P < 0.0001$; Mann-Whitney test [Fig. 3B]). When we examined the $CD3^+$ $CD8^+$ IFN-γ^+ T cell response of YFLAV (YF17D) vaccinees to the other heterologous MPs, we found significantly lower expression for the CD69 and CD137 markers (YFV-specific versus other flavivirus MPs, $P = 0.0006$ and 0.0038, respectively), while a nonsignificant trend was observed for CD40L (YFV-specific versus other flavivirus MPs, $P = 0.1784$) (Fig. 3B). Overall, these data suggest that CD8 T cells that recognize cross-reactive heterologous sequences have a trend for lower expression of CD69, CD137, and CD40L activation markers.

Monovalent DLAV vaccination induces CD8 T cell cross-reactivity against other DENV serotypes but is limited against other flaviviruses. The data presented above suggest that DENV or YFV vaccination induces responses that are only marginally cross-reactive with other flavivirus species in terms of both magnitude and activation capacity. These data were obtained utilizing epitope MPs containing hundreds of different peptides. To characterize the phenomenon by a different approach, we analyzed responses against representative individual epitopes. For this purpose, we derived epitope-specific short-term T cell lines (TCLs) by stimulating PBMCs for 14 days with the homologous peptide. Their antigen sensitivity was quantified by determining dose-response curves. In parallel, we determined the reactivity of these TCLs to peptides corresponding to the homologous epitope in parallel to their sensitivity to heterologous corresponding sequences derived from the other flaviviruses studied here. Comparing the dose response of the homologous epitope with that of the heterologous peptides from the various flaviviruses allowed for the quantification of relative potencies.

We first determined the level of cross-reactivity in six different TCLs from four monovalent DLAV vaccinees (immunized with either DEN1Δ30 or DEN3Δ30,31). To ensure that the epitopes studied were representative of *in vivo* vaccination, we selected PBMCs and epitopes from donors that we had previously screened in *ex vivo* IFN-γ enzyme-linked immunosorbent spot (ELISPOT) assays, following vaccination with specific monovalent DLAV vaccines (32). Specifically, we selected three epitopes derived from NS5 protein, two derived from NS3 and one derived from M protein. The homologous as well as heterologous peptides corresponding to the other three DENV serotypes and YFV, ZIKV, JEV, and WNV sequences were tested at six concentrations to assess the relative potencies (Fig. 4A to F). As expected in all cases, responses to the homologous peptides were the most dominant. If responses to any of the heterologous peptides were detected, we calculated the fold difference in antigen sensitivity compared to the homologous peptide.

Of 42 heterologous peptides tested, high cross-reactivity (defined as reactivity within 10-fold of the homologous peptide) was detected in 7 of them (17% of the total). No instance of cross-reactivity was detected in the high and moderate potency ranges (1- to 100-fold-lower response than to homologous peptide), while in four heterologous peptides (10% of the total), reactivity in the low-potency range (101- to 1,000-



G

Fold Change	1-10	10.1-100	101-1000	1001-10000	>10000 or negative
DENV 1	4	0	0	0	0
DENV 2	1	0	1	1	3
DENV 3	0	0	1	1	0
DENV 4	2	0	2	1	1
ZIKV	0	0	0	2	4
YFV	0	0	0	0	6
JEV	0	0	0	1	5
WNV	0	0	0	1	5

FIG 4 Relative potencies of homologous and heterologous flavivirus peptides for CD8$^+$ T cells derived from monovalent DENV vaccination. Spot-forming cells (SFC) per million CD8 cells are plotted for six TCLs stimulated with each peptide at six concentrations after 14 days of *in vitro* expansion and derived from four DENV monovalent vaccinees. Specific peptide responses from vaccinees are shown in panels A to D (DEN3Δ30,31) and E and F (DEN1Δ30,31); panels B and C show results for independent TCLs specific for the same epitopes but derived from two different donors, respectively. (G) Summary of the patterns of the relative potencies of heterologous peptides compared to the homologous immunizing sequence. Relative potency was calculated for each homologous/heterologous peptide combination based on observed dose responses by recording which peptide dose would give equivalent SFC/10^6 values. The number of instances where the heterologous sequences were associated with a relative potency of 1 to 10 (high), 10.1 to 100 (intermediate), >100.1 to 1,000 (weak), 1,000.1 to 10,000 (very weak), or>10,000 or negative is shown.

fold-lower response than to homologous peptides) was detected. Finally, 7 heterologous peptides (17% of the total) were associated with very low potency (1,001- to 10,000-fold-lower response than to homologous peptides) and 24 heterologous peptides (57%) were negative, defined as a >10,000-fold-lower potency. DENV1 and DENV4 sequences were cross-recognized in the highest number of instances, followed by DENV2. DENV3 showed the weakest relative potency range across all the DENV serotypes. Cross-reactive responses against heterologous ZIKV, JEV, and WNV peptides were detected in one peptide each, although with low relative potency (101- to 1,000-fold range), and in all remaining instances, no cross-reactivity was detected. Heterologous YFV peptides did not stimulate cross-reactive T cell responses in all the instances analyzed. From the summary data in Fig. 4G, we conclude that while a degree of cross-reactivity between different DENV serotypes was detected, cross-reactivity with other flaviviruses was limited or, in the case of YFV, totally absent.

YFV vaccination induces minimal CD8 T cell cross-reactivity against other flaviviruses. To generalize and expand these findings, we performed similar experiments utilizing PBMCs from donors vaccinated with the YF17D vaccine, and epitopes previously identified in *ex vivo* IFN-γ ELISPOT assays, in the context of an epitope identification study (D. Weiskopf, A. Grifoni, and A. Sette, unpublished data). As described above, PBMCs were expanded with YFV-specific epitopes for 14 days. The homologous and heterologous peptides were assayed over a 100,000-fold dose range to assess their relative potencies. Figure 5A to H show results from eight different TCLs derived from six different YF17D vaccinees; five of the original epitopes were derived from NS5 protein, and one each was derived from M, NS2B, and NS4B proteins. For one TCL shown in Fig. 5C, cross-reactive responses were detected against all heterologous flavivirus sequences. In this case, the sequence homology between all peptides tested was 90% or more. For the TCLs shown in Fig. 5B and H, cross-reactivities with other flaviviruses sequences, such as JEV, WNV, and DENV2, were detected.

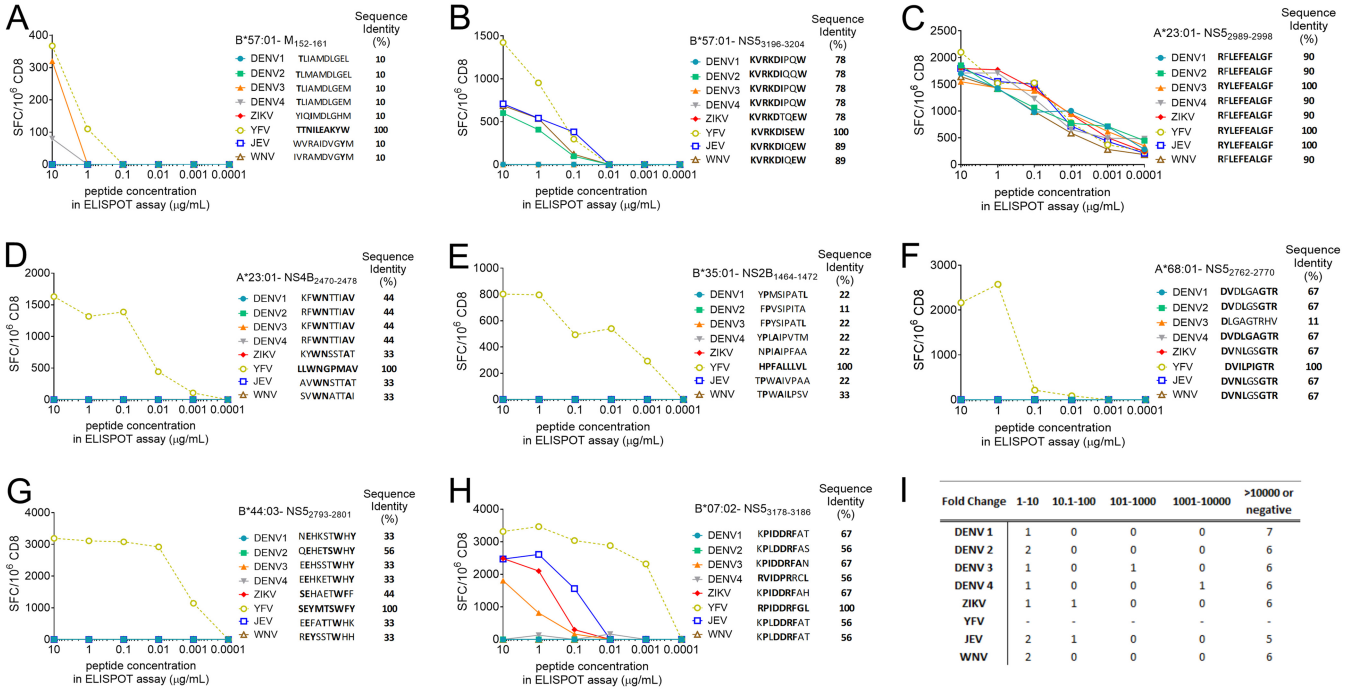


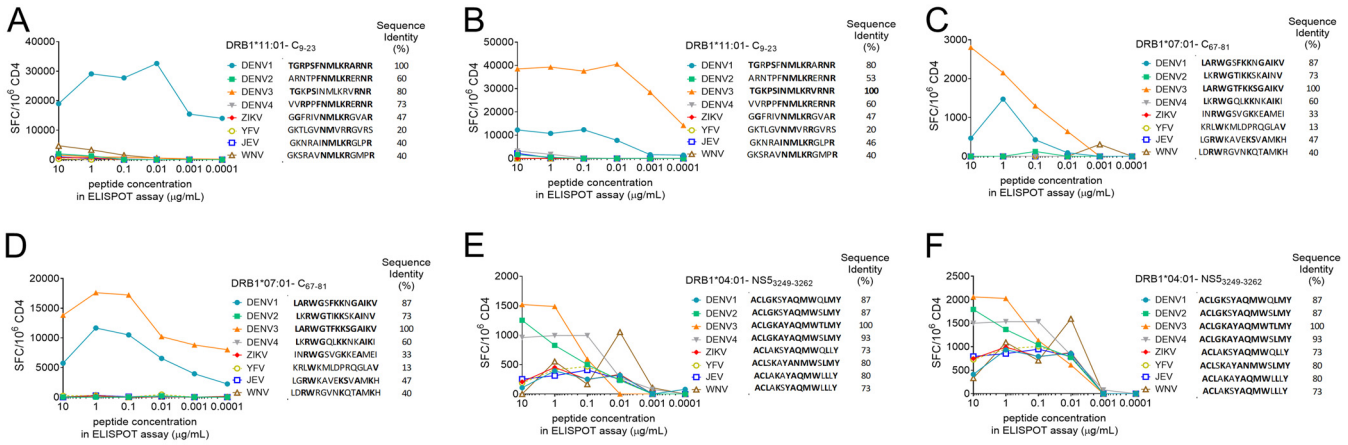
FIG 5 Relative potencies of homologous and heterologous flavivirus peptides for CD8$^+$ T cells derived from YF17D vaccination. (A to H) Spot-forming cells per million CD8 cells are plotted for TCLs stimulated with each peptide at six concentrations after 14 days of *in vitro* expansion derived from six YF17D vaccinees. (I) Summary of the patterns of the relative potency of heterologous peptides compared to the homologous immunizing sequence. Relative potency was calculated for each homologous/heterologous peptide combination based on observed dose responses by recording which peptide dose would give equivalent SFC/10^6 values. The number of instances where the heterologous sequences were associated with a relative potency of 1 to 10 (high), 10.1 to 100 (intermediate), >101 to 1,000 (weak), 1,001 to 10,000 (very weak), or >10,000 or negative is shown.

Of the 56 heterologous peptides tested, high cross-reactivity (defined as reactivity within a 10-fold of the response induced by the homologous peptide) was detected in 10 (18% of the total). Cross-reactivity in the 10.1- to 100-fold moderate relative potency range was detected in two heterologous peptides (4% of the total), and only one heterologous peptide was found in the lowest 101- to 1,000-fold and 1,001 to 10,000 relative potency range (2% of the total in both cases). No cross-reactivity was detected for 42 of the heterologous peptides, corresponding to 75% of the total (Fig. 5). In conclusion, CD8 T cells induced by the YF17D vaccine showed minimal cross-reactivity against other flaviviruses, with the DENV serocomplex being the least cross-recognized flavivirus.

Vaccine-induced CD4 T cell cross-reactivity is even more limited than CD8. Since live attenuated vaccines induce both CD8 and CD4 T cell responses, we asked next whether we could detect cross-reactivity between flaviviruses at the level of CD4 T cell responses. Following the same strategy as described above, we expanded DLAV- and YFV-specific CD4 T cell lines for 14 days and tested homologous and heterologous sequences to assess relative potencies.

We derived six different TCLs from two monovalent DENV vaccinees (DEN1Δ30 and DEN3Δ30,31) representing three epitopes recognized in each of the two vaccinees (Fig. 6A to F), four of them derived from capsid protein and two derived from NS5.

Of the 45 heterologous peptides tested, high cross-reactivity (defined as reactivity within 10-fold of the homologous peptide) was detected in only 2 (4% of the total). More limited cross-reactivity in the moderate (10.1- to 100-fold-lower) potency range was detected in four heterologous peptides (9% of the total) and was detected in the low (101- to 1,000-fold) range in two heterologous peptides (4% of the total). Finally, the majority of heterologous peptides (32 out of 45 [71% of the total]) were in the very low (1,001- to 10,000-fold lower) relative potency range, and no cross-reactivity was detected for 5 of the heterologous peptides, corresponding to 11% of the total



G

Fold Change	1-10	10.1-100	101-1000	1001-10000	>10000 or negative
DENV 1	0	1	0	4	0
DENV 2	1	0	2	4	0
DENV 3	1	0	0	2	0
DENV 4	0	2	0	4	0
ZIKV	0	0	0	4	2
YFV	0	0	0	4	2
JEV	0	1	0	5	0
WNV	0	0	0	5	1

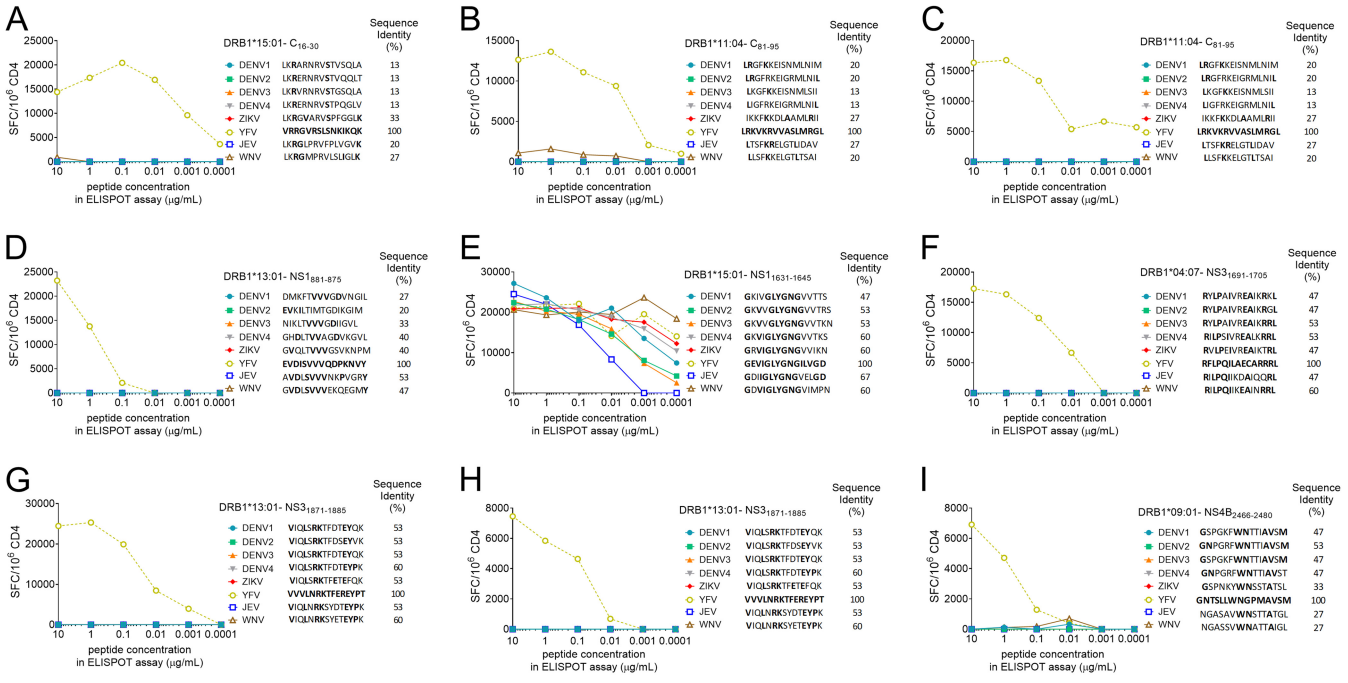
FIG 6 Relative potencies of homologous and heterologous flavivirus peptides for CD4$^+$ T cells derived from monovalent DENV vaccination. Spot-forming cells per million CD4 cells are plotted for seven TCLs stimulated with each peptide at six concentrations after 14 days of *in vitro* expansion and derived from six DENV monovalent vaccinees. Specific peptide responses from vaccinees are shown in panel A (DEN1Δ30,31) and panels B to F (DEN3Δ30,31); panels A and B and E and F show independent TCLs specific for the same epitopes but derived from two different donors, respectively. (G) Summary of the patterns of the relative potency of heterologous peptides compared to the homologous immunizing sequence. Relative potency was calculated for each homologous/heterologous peptide combination based on observed dose responses by recording which peptide dose would give equivalent SFC/10^6 values. The number of instances where the heterologous sequences were associated with a relative potency of 1 to 10 (high), 10.1 to 100 (intermediate), >101 to 1,000 (weak), 1,001 to 10,000 (very weak), or >10,000 or negative is shown.

(Fig. 6G). In conclusion, an even lower apparent degree of cross-reactivity between different DENV serotypes was detected within the CD4 compartment than within the CD8 compartment, and the cross-reactivity with other flaviviruses was very limited for both CD4 and CD8 T cells.

Next, we derived TCLs derived from seven different YF17D vaccinees (Fig. 7A to I). In 7 of the 63 heterologous peptides considered (12% of the total), high cross-reactivity in the 1- to 10-fold range was observed, all to be ascribed to a single peptide sharing the amino acid core GLYGNG across the different flavivirus species (Fig. 7E). Three additional heterologous peptides (0.5% of the total) showed a minimal level of cross-reactivity, all corresponding to the heterologous WNV sequence with relative potency levels within the 1001- to 10,000-fold range, while the vast majority of the heterologous peptides (82% of the total) did not show any cross-reactivity. Overall, these data demonstrate limited CD4 cross-reactivity against other flaviviruses after YF17D vaccination. In conclusion, our data demonstrate that while vaccination with monovalent DLAV vaccines induced some CD8 and CD4 T cells cross-reactivity, mostly against the other DENV serotypes, the T cell cross-reactivity induced by the YF17D vaccine was limited and mostly absent.

DISCUSSION

Flaviviruses such as DENV, ZIKV, JEV, WNV, and YFV are highly homologous to each other and often circulate in the same geographical regions. The cross-reactivity is expected to be more pronounced in the case of the TV005 vaccine since in this case it was shown that TV005 focuses the responses on conserved (and thereby by definition cross-reactive) epitopes. We studied the level of cross-reactivity of T cells induced by natural infection and vaccination with live attenuated flavivirus vaccines. We demonstrate that broad cross-reactivity among sequences of different flaviviruses exists and is largely associated with the recognition of sequences derived from different DENV



J

Fold Change	1-10	10.1-100	101-1000	1001-10000	>10000 or negative
DENV 1	1	0	0	0	9
DENV 2	1	0	0	0	9
DENV 3	1	0	0	0	9
DENV 4	1	0	0	0	9
ZIKV	1	0	0	0	9
YFV	-	-	-	-	-
JEV	1	0	0	0	9
WNV	1	0	0	3	5

FIG 7 Relative potencies of homologous and heterologous flavivirus peptides for CD4$^+$ T cells derived from YF17D vaccination. (A to I) Spot-forming cells per million CD4 cells are plotted for TCLs stimulated with each peptide at six concentrations after 14 days of *in vitro* expansion derived from nine YF17D vaccinees. Specific peptide responses from YF17D vaccinees are shown. (J) Summary of the patterns of the relative potency of heterologous peptides compared to the homologous immunizing sequence. Relative potency was calculated for each homologous/heterologous peptide combination based on observed dose responses by recording which peptide dose would give equivalent SFC/10^6 values. The number of instances where the heterologous sequences were associated with a relative potency of 1 to 10 (high), 10.1 to 100 (intermediate), >101 to 1,000 (weak), 1,001 to 10,000 (very weak), or >10,000 or negative is shown.

serotypes. Cross-reactivity among different flavivirus species was limited and was associated with responses of lower frequency and magnitude. It was further found that T cells activated from cross-reactive sequences displayed lower levels of expression of common activation markers and not increased cytokine secretion.

One limitation of this study is that the overall *ex vivo* cross-reactive responses were measured after stimulation to selected predicted epitope MPs. We cannot exclude that we missed potential cross-reactive epitopes by mostly including bioinformatically defined epitopes. However, this does not affect the findings at the single-epitope level, where we tested the cross-reactive potential of experimentally defined epitopes.

It should be pointed out that these results are meant not to be an absolute quantitative determinant but rather to provide multiple examples of responses observed in different peptides, different donors, and different HLAs.

These data provide further insights regarding what level of sequence homology is generally associated with potential cross-reactivity.

The data have implications for understanding immunity elicited by vaccination and/or natural infection. In particular, they suggest that the use of YFV as a backbone to engineer live vaccines to deliver E and prM proteins from other flaviviruses is not likely to generate optimal T cell responses against other flaviviruses.

The cross-reactivity between DENV- and YFV-derived epitopes observed was fairly limited. The absence of the NS and C DENV proteins, which are immunodominant for CD4 and CD8 responses in Dengvaxia (37), combined with the limited cross-reactivity

observed in this study, might contribute to the relatively low level of efficacy observed for this vaccine. This is in agreement with a murine model of heterologous flavivirus infection in which previous exposure to YF did not provide cross-reactive functional protection against DENV1 challenge (11).

Traditionally, flaviviruses have been subdivided into so-called serocomplexes, comprising members that are cross-neutralized by polyclonal sera. This classification largely correlates with the amino acid sequence identity of E and led to the establishment of the DENV serocomplex (consisting of DENV serotypes 1 to 4) and the JEV serocomplex (also including WNV) (38). Zika virus is more closely related to DENV than to the JE virus serocomplex or to YFV, which is almost as distantly related to the other mosquito-borne flaviviruses as it is to the tick-borne viruses (38). In accordance with this general overall level of sequence homology, we have observed the highest cross-reactivity within the DENV serocomplex after monovalent DENV vaccination. Thus, while T cell cross-reactivity is appreciable across different serocomplexes/serotypes, T cell cross-reactivity is limited across different flavivirus species.

T cells recognize peptide epitopes derived from the original priming antigen and/or vaccine and are reactivated in subsequent encounters with the same exact epitopes but also from closely related epitopes. The concept of original antigenic sin, originally described for antibody responses in influenza (39), implies that the pathogen strain shapes subsequent responses to other influenza virus strains. In the case of DENV, the concept of original sin was postulated to contribute to immunopathology (40, 41), but later studies showed that while previous exposure to different DENV serotypes influenced the repertoire of responding T cells, both in humans (22, 42) and in mice (43), the effect was mostly reflected in increases in cross-reactive T cells recognizing conserved epitopes, and cross-reactive T cells were associated with protection in murine models of DENV infection (19, 44). Consistent with this notion, we have previously shown that the simultaneous administration of all four monovalent DENV vaccine strains leads to the induction of highly conserved sequences against all four DENV serotypes (32).

While heterologous sequences were generally associated with incomplete cross-reactivity in this study, this does not rule out a contribution of cross-reactive responses in influencing disease and vaccination outcomes. Indeed, in a murine model of DENV infection, it has been shown that despite being associated with lower-magnitude responses, cross-reactive CD8 T cell epitopes can still contribute to protection by lowering the viral titer in DENV-infected mice (9, 16, 17, 19). We have also shown that DENV preexposure influences T cell responses against the highly homologous ZIKV in both human and murine systems (22, 44).

In our studies, not only was the degree of cross-reactive recognition of sequences derived from other flaviviruses limited, but also the T cell activation induced by the cross-reactive species was suboptimal, resulting in lower expression of several activation markers. This is consistent with the original description by Evavold and Allen (45) of the phenomenon of altered peptide ligands (APL), epitope variants carrying one or more substitutions. A large body of literature suggests that APL can trigger incomplete T cell activation and clarified some of the mechanism involves in the effect, as ascribed to lower levels of Zap70 phosphorylation and other T cell receptor (TCR) signaling alterations (46). Thus, it seems that epitope variants in some cases are fully cross-reactive while in other cases are incompletely activated. We saw no evidence of increased cytokine production as suggested by other studies hypothesizing a cytokine storm induced by heterologous sequences as a mechanism of DENV pathogenesis (40, 41).

Our data also provide insights regarding what degree of sequence homology is necessary for cross-reactivity, at the level of CD4 and CD8 T cell responses. Specifically, CD8 T cell cross-reactivity was detected in 9 out of 9 instances of heterologous sequences that had one substitution (about 90% sequence identity for 9/10-mers) compared to the immunizing epitope. Cross-reactivity was detected in 6 out of 9 instances of heterologous sequences that had two substitutions (about 80% sequence identity for 9/10-mers). Cross-reactivity was detected in 5 out of 15 heterologous

sequences that had three substitutions (about 70% sequence identity for 9/10-mers). Finally, cross-reactivity was detected in only 5 out of 61 instances of heterologous sequences that had four or more substitutions (less than 67% sequence identity). Thus, 80% of cross-reactive responses were associated with 67% or more sequence identity.

This is in agreement with our previous results, where we found that CD8 T cell cross-reactivity was typically detected for heterologous epitopes that shared 70% or higher sequence identity (42) and substitution of 1 or 2 amino acids marked the threshold for CD8 epitopes (43). In contrast, in the case of CD4 responses, no clear pattern could be discerned, with peptides sharing as little as 50% sequence identity being associated with high cross-reactivity. However, the analysis was difficult because the overall degree of identity among the peptides tested was below 60% except for epitopes within the dengue virus complex. While the molecular mechanism of this difference is not addressed by the current study, this might be related to the fact that in the case of CD4 responses, each antigenic 15-mer epitope might bind in several different registers, and as a result, the degree of homology of the central core region recognized by the CD4 response might be higher than what is recorded for the overall peptide.

In conclusion, the results of this study emphasize the need to accurately assess T cell responses and the potential to cross-react with related pathogens in the context of vaccine development and also suggest that when vaccine vectors with significant homology to the vaccine target are used, vaccine vector responses should also be evaluated.

MATERIALS AND METHODS

Epitope MP design and homology analyses. The epitope CD8 megapool (MP) was produced by sequential lyophilization of flavivirus-specific epitopes as we previously described; in particular, the DENV CD8 MP has been previously generated and validated in DENV-exposed individuals from different geographical areas (29, 47).

Flavivirus-specific epitopes were retrieved by querying the Immune Epitope Database (IEDB) (48) utilizing the following search parameters positive assay only, no B cell assays, no major histocompatibility complex (MHC) ligand assay, host *Homo sapiens*, and MHC restriction class I. In the cases of ZIKV, YFV, JEV, and WNV, experimentally defined epitopes were supplemented by the predicted epitopes using TepiTool (49) algorithm. For this purpose, we used previously published consensus sequences for ZIKV, YFV, JEV, and WNV (35).

For yellow fever virus, we supplemented the published consensus sequence with the sequence of the YF17D vaccine strain (UniProt accession number P03314) and the sequence of a virus isolate derived from the recent outbreak in Brazil (GenBank accession number ARM37843.1) (50).

To perform the epitope prediction, a previously described method to predict the most frequent A and B alleles was considered (51), and predictions were performed for both 9-mers and 10-mers with a consensus percentile rank cutoff of $\leq$1.5. A subsequent HLA allele-specific filter was applied based on the percentile cutoff based on our studies performed on DENV infection (9, 29). When the HLA allele considered was not available, the median of the known alleles was used (as summarized in Table 3).

The resulting peptides were then clustered using the IEDB cluster 2.0 tool and applying the IEDB recommended method (i.e., cluster break method) with a 70% cutoff for sequence identity (52, 53). Peptides were synthesized as crude material (A&A, San Diego, CA), resuspended in dimethyl sulfoxide (DMSO), pooled according to each flavivirus MP composition, and, finally, sequentially lyophilized (47).

Homology analyses to dissect the homology level between each MP and the viral consensus sequences have been performed using the Immunobrowser tool (54). For each MP, the fraction of peptides with a sequence identity of $\geq$70% with each flavivirus consensus sequence was calculated. In the context of the DENV CD8 MP, homology analyses were carried out in each DENV consensus sequence calculated per serotype, and then the maximum value of homology obtained across the four serotypes was used for each peptide analyzed.

Study subjects. PBMCs from healthy adult blood bank donors in areas where DENV is endemic were collected anonymously from National Blood Center, Ministry of Health, Colombo, Sri Lanka, and from the Nicaraguan National Blood Center, Managua, Nicaragua, as previously described (27).

In Nicaragua, samples were collected in 2015 to 2016, prior to the introduction of ZIKV into the Americas. All protocols were approved by the institutional review boards of both the La Jolla Institute for Immunology (LJI) and Medical Faculty, the University of California, Berkeley, the Nicaraguan Ministry of Health, and the University of Colombo (serving as an NIH-approved internal review board [IRB] for Genentech). Blood collection and processing were performed in the two cohorts as we previously described (27, 55).

The yellow fever live attenuated vaccine (YF17D) cohort and the flavivirus naive cohorts consist of healthy donors: adult male and nonpregnant female volunteers, 18 to 50 years of age, that were enrolled

TABLE 3 List of cutoffs used per HLA class I based on previous DENV studies

HLA	% cutoff
A*01:01	0.75
A*02:01	0.4
A*02:06	1.05
A*03:01	0.35
A*23:01	1.1
A*24:02	1
A*26:01	0.15
A*31:01	0.85
A*33:01	0.85
A*68:01	0.45
A*68:02	1.5
B*07:02	0.35
B*15:01	0.7
B*35:01	1
B*40:01	0.25
B*44:02	0.4
B*44:03	0.4
B*51:01	0.6
B*53:01	0.7
B*57:01	0.25
B*58:01	0.3
Unknown	0.6

and either vaccinated with YF17D ($n = 15$) or not (flavivirus naive cohort; $n = 10$) under the LJI program VD-101.

PBMCs derived from flavivirus-naive and YF17D cohorts were processed at LJI by density gradient sedimentation using Ficoll-Paque (Lymphoprep; Nycomed Pharma, Oslo, Norway). Isolated PBMCs were cryopreserved in heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT), containing 10% DMSO (Gibco) and stored in liquid nitrogen until use in the assays.

The dengue fever live attenuated vaccinees (TV005) consist of healthy donors vaccinated with one or four of the dengue live attenuated viruses (DEN1Δ30, DENV4Δ30, DEN3Δ30/31, and DEN2/4Δ30), as previously reported (36, 56–58). Clinical trials for those vaccinations are described at ClinicalTrials.gov under numbers NCT01084291, NCT01073306, NCT00831012, NCT00473135, NCT00920517, NCT00831012, and NCT01072786.

Both vaccinee cohorts were analyzed 6 to 12 months after the initial vaccination.

Flow cytometry. Cells were cultured in the presence of either the DENV, YFV, ZIKV, JEV, or WNV MP (1 μg/ml) or DMSO (0.1%) as negative control together with brefeldin A (BD GolgiPlug; BD Biosciences) for 6 h. After stimulation, cells were stained with surface markers for 30 min at 4°C, followed by fixation with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) at 4°C for 10 min. Intracellular staining was performed at room temperature (RT) for 30 min after cells permeabilization with saponin, as previously described (59, 60). Detailed information on all the antibodies used for flow cytometry experiments in this study can be found in Table 4.

Surface marker proteins and intracellular cytokine responses were quantified via flow cytometry (LSRII; BD Biosciences) and analyzed using FlowJo software version 10.5.3 (TreeStar Inc., Ashland, OR). The gating strategy is schematically represented in Fig. 8. Within the $CD3^+$ $CD8^+$ subset of lymphocytes, the differences in the magnitude of response between MP stimuli were assessed based on IFN-γ^+ frequency of parent percentage. The quality of the response was investigated by comparing the intracellular staining of the CD40L, CD69, and CD137 markers within the entire CD8 population and within the $CD8^+$

TABLE 4 Antibody panel used in flow cytometry experiments to identify both magnitude and quality of $CD8^+$ T cell response and relevant subpopulations[a]

Antibody	Fluorochrome	Vol (ml)/test	Vendor	Catalog	Clone
CD4	APC ef 780	1	eBioscience	47-0049-42	RPA-T4
CD3	AF700	2	Biolegend	317340	OKT3
CD8	BV650	2	BioLegend	301042	RPA-T8
CD14	V500	2	BD Biosciences	561391	M5E2
CD19	V500	2	BD Biosciences	561121	HIB19
Fixability dye	ef506	1 μl/ml of master mix	eBioscience	65-0866-18	NA
IFN-γ	FITC	1	eBioscience	11-7319-82	4S.B3
CD154 (CD40L)	PE	2	eBioscience	12-1548-42	24-31
CD69	PE Cy7	2	eBioscience	25-0699-42	FN50
CD137(4-1BB)	APC	2	BioLegend	309810	4B4-1

[a]Abbreviations: APC, allophycocyanin; PE, phycoerythrin; FITC, fluorescein isothiocyanate; NA, not applicable.

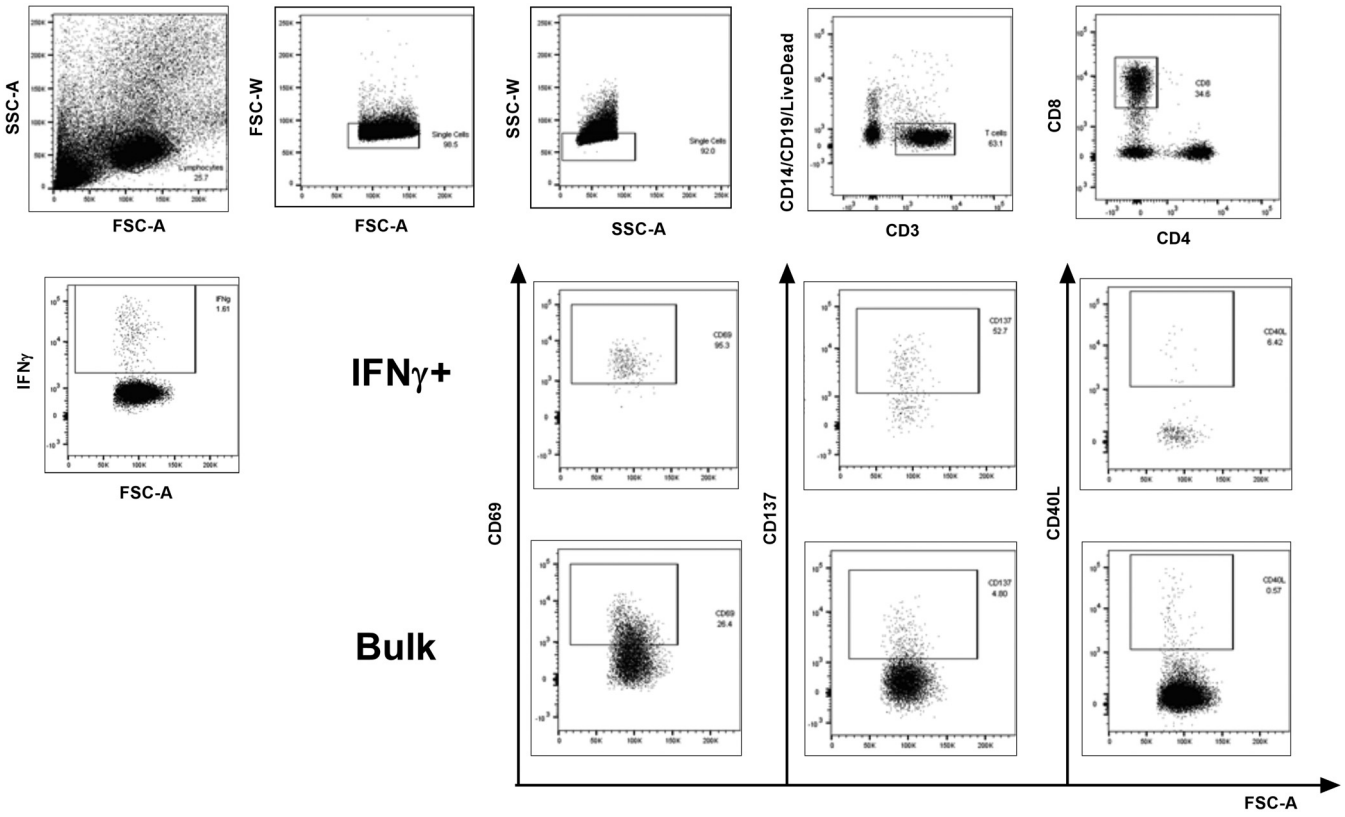


FIG 8 Gating strategy. To examine IFN-γ production in $CD8^+$ T cells, lymphocytes were gated from the whole PBMCs on forward scatter A (FSC-A) and side scatter A (SSC-A) axes, followed by exclusion of outlying data points on FSC-W and SSC-W parameters. Cells positive for viability stain, as well as those found to be $CD3^-$, were excluded. Of those remaining, cells were separated based on CD4 and CD8 expression parameters and CD8 were exclusively investigated. Intracellular expression levels of T cell activation markers CD69, CD137, and CD40L were examined in whole CD8, bulk, and $CD8^+$ IFN-γ^+ cells. Samples were acquired on an LSRII (BD Biosciences, San Diego, CA).

IFN-γ^+ subsets using background-subtracted values and a 0.03 cutoff for positivity for the different stimuli.

ELISPOT assays on short-term TCL to quantitate the antigen dose responses. Short-term T cell lines (TCLs; 14 days) were set up using donors previously vaccinated with monovalent DENV or YFV vaccines. Cells were expanded using specific DENV epitopes corresponding to the original vaccination and identified in previous studies (32). YFV epitopes were identified using the same approach in YFLAV donors. Cells were expanded using specific DENV/YFV epitopes corresponding to the original vaccination identified using the same approach as previously described (24). After 14 days, IFN-γ ELISPOT assays were performed as previously described (9, 27, 28). Briefly, each TCL was tested with the epitope derived from the immunizing vaccine and peptides corresponding to analogous sequences from the different DENV serotypes or other flaviviruses (YFV, ZIKV, JEV, and WNV) in triplicate. Each peptide was tested at six different concentrations (10 μg/ml, 1 μg/ml, 0.1 μg/ml, 0.01 μg/ml, 0.001 μg/ml, and 0.0001 μg/ml). Cells were stimulated for 20 h at 37°C and 5% CO_2 at a concentration of 1×10^5 cells/ml of medium on plates previously coated with anti-human IFN-γ (monoclonal antibody [MAb] 1-D1K; Mabtech, Stockholm, Sweden). Cells were then discarded and plates were further incubated with biotinylated IFN-γ antibody (MAb 7-B6-1; Mabtech) and incubated for 2 h at 37°C. Avidin peroxidase complex (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) and 3′-amino-9-ethyl carbazole (AEC tablets; Sigma, St. Louis, MO) were further used to develop the plate. Image analysis was performed using a KS-ELISPOT reader (Zeiss, Munich, Germany).

Statistics. Statistical analyses were performed using GraphPad Prism (San Diego, CA). Specifically, the analysis of the responses for different cohorts against the same stimuli was performed using unpaired, nonparametric Mann-Whitney test, while to compare the same cohort's response to different stimuli, a paired, nonparametric Wilcoxon test was used. The relative-potency analyses were performed by determining the dose response to each homologous peptide required to achieve a level of response that is comparable to the dose response of the immunizing epitope and calculate the corresponding fold difference in terms of antigen sensitivity determined by measuring the shift in dose response observed in the *x* axis, as previously described (61).

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health contracts HHSN272200900042C and HHSN27220140045C to A. Sette, 75N9301900065 to A. Sette and D. Weiskopf,

and P01AI106695 to E. Harris. Additional support was provided by contract HHSN272200900045C to E. Harris. The TV003/005 clinical trials were funded by contract HHSN272200900010C of the Intramural Research Program of the NIH, NIAID.

D.W. has been on the scientific advisory boards on dengue vaccine evaluation for Merck and Takeda. All other authors declare no conflict of interest.

A. Grifoni, H. Voic, and C. K. Kidd performed experiments, reviewed data, and planned the experimental strategy. S. K. Dhanda and E. Harris performed and assisted in the bioinformatics analyses. A. P. Durbin, S. Whitehead, S. A. Diehl, J. D. Brien, A. Balmaseda, E. Harris, A. Stryhn, and A. D. De Silva provided samples and sample information. A. Grifoni, D. Weiskopf, and A. Sette conceived and directed the study and wrote the manuscript. All authors have critically read and edited the manuscript.

REFERENCES

1. Daep CA, Muñoz-Jordán JL, Eugenin EA. 2014. Flaviviruses, an expanding threat in public health: focus on dengue, West Nile, and Japanese encephalitis virus. J Neurovirol 20:539–560. https://doi.org/10.1007/s13365-014-0285-z.
2. Hassert M, Brien JD, Pinto AK. 2019. Mouse models of heterologous flavivirus immunity: a role for cross-reactive T cells. Front Immunol 10:1045. https://doi.org/10.3389/fimmu.2019.01045.
3. Katzelnick LC, Harris E, Participants in the Summit on Dengue Immune Correlates of Protection. 2017. Immune correlates of protection for dengue: state of the art and research agenda. Vaccine 35:4659–4669. https://doi.org/10.1016/j.vaccine.2017.07.045.
4. Halstead SB. 2014. Dengue antibody-dependent enhancement: knowns and unknowns. Microbiol Spectr 2:AID-0022-2014. https://doi.org/10.1128/microbiolspec.AID-0022-2014.
5. Katzelnick LC, Gresh L, Halloran ME, Mercado JC, Kuan G, Gordon A, Balmaseda A, Harris E. 2017. Antibody-dependent enhancement of severe dengue disease in humans. Science 358:929–932. https://doi.org/10.1126/science.aan6836.
6. Nivarthi UK, Kose N, Sapparapu G, Widman D, Gallichotte E, Pfaff JM, Doranz BJ, Weiskopf D, Sette A, Durbin AP, Whitehead SS, Baric R, Crowe JE, Jr, de Silva AM. 2017. Mapping the human memory B cell and serum neutralizing antibody responses to dengue virus serotype 4 infection and vaccination. J Virol 91:e02041-16. https://doi.org/10.1128/JVI.02041-16.
7. Swanstrom JA, Nivarthi UK, Patel B, Delacruz MJ, Yount B, Widman DG, Durbin AP, Whitehead SS, De Silva AM, Baric RS. 2019. Beyond neutralizing antibody levels: the epitope specificity of antibodies induced by National Institutes of Health monovalent dengue virus vaccines. J Infect Dis 220:219–227. https://doi.org/10.1093/infdis/jiz109.
8. VanBlargan LA, Mukherjee S, Dowd KA, Durbin AP, Whitehead SS, Pierson TC. 2013. The type-specific neutralizing antibody response elicited by a dengue vaccine candidate is focused on two amino acids of the envelope protein. PLoS Pathog 9:e1003761. https://doi.org/10.1371/journal.ppat.1003761.
9. Weiskopf D, Angelo MA, de Azeredo EL, Sidney J, Greenbaum JA, Fernando AN, Broadwater A, Kolla RV, De Silva AD, de Silva AM, Mattia KA, Doranz BJ, Grey HM, Shresta S, Peters B, Sette A. 2013. Comprehensive analysis of dengue virus-specific responses supports an HLA-linked protective role for CD8+ T cells. Proc Natl Acad Sci U S A 110:E2046–E2053. https://doi.org/10.1073/pnas.1305227110.
10. Weiskopf D, Bangs DJ, Sidney J, Kolla RV, De Silva AD, de Silva AM, Crotty S, Peters B, Sette A. 2015. Dengue virus infection elicits highly polarized CX3CR1+ cytotoxic CD4+ T cells associated with protective immunity. Proc Natl Acad Sci U S A 112:E4256–E4263. https://doi.org/10.1073/pnas.1505956112.
11. Saron WAA, Rathore APS, Ting L, Ooi EE, Low J, Abraham SN, St John AL. 2018. Flavivirus serocomplex cross-reactive immunity is protective by activating heterologous memory CD4 T cells. Sci Adv 4:eaar4297. https://doi.org/10.1126/sciadv.aar4297.
12. Montoya M, Collins M, Dejnirattisai W, Katzelnick LC, Puerta-Guardo H, Jadi R, Schildhauer S, Supasa P, Vasanawathana S, Malasit P, Mongkolsapaya J, de Silva AD, Tissera H, Balmaseda A, Screaton G, de Silva AM, Harris E. 2018. Longitudinal analysis of antibody cross-neutralization following Zika virus and dengue virus infection in Asia and the Americas. J Infect Dis 218:536–545. https://doi.org/10.1093/infdis/jiy164.
13. Saito Y, Moi ML, Takeshita N, Lim CK, Shiba H, Hosono K, Saijo M, Kurane I, Takasaki T. 2016. Japanese encephalitis vaccine-facilitated dengue virus infection-enhancement antibody in adults. BMC Infect Dis 16:578. https://doi.org/10.1186/s12879-016-1873-8.
14. Halstead SB, Rojanasuphot S, Sangkawibha N. 1983. Original antigenic sin in dengue. Am J Trop Med Hyg 32:154–156. https://doi.org/10.4269/ajtmh.1983.32.154.
15. Mongkolsapaya J, Dejnirattisai W, Xu XN, Vasanawathana S, Tangthawornchaikul N, Chairunsri A, Sawasdivorn S, Duangchinda T, Dong T, Rowland-Jones S, Yenchitsomanus PT, McMichael A, Malasit P, Screaton G. 2003. Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever. Nat Med 9:921–927. https://doi.org/10.1038/nm887.
16. Yauch LE, Prestwood TR, May MM, Morar MM, Zellweger RM, Peters B, Sette A, Shresta S. 2010. CD4+ T cells are not required for the induction of dengue virus-specific CD8+ T cell or antibody responses but contribute to protection after vaccination. J Immunol 185:5405–5416. https://doi.org/10.4049/jimmunol.1001709.
17. Yauch LE, Zellweger RM, Kotturi MF, Qutubuddin A, Sidney J, Peters B, Prestwood TR, Sette A, Shresta S. 2009. A protective role for dengue virus-specific CD8+ T cells. J Immunol 182:4865–4873. https://doi.org/10.4049/jimmunol.0801974.
18. Angelo MA, Grifoni A, O'Rourke PH, Sidney J, Paul S, Peters B, de Silva AD, Phillips E, Mallal S, Diehl SA, Kirkpatrick BD, Whitehead SS, Durbin AP, Sette A, Weiskopf D. 2017. Human CD4+ T cell responses to an attenuated tetravalent dengue vaccine parallel those induced by natural infection in magnitude, HLA restriction, and antigen specificity. J Virol 91:e02147-16. https://doi.org/10.1128/JVI.02147-16.
19. Elong Ngono A, Chen HW, Tang WW, Joo Y, King K, Weiskopf D, Sidney J, Sette A, Shresta S. 2016. Protective role of cross-reactive CD8 T cells against dengue virus infection. EBioMedicine 13:284–293. https://doi.org/10.1016/j.ebiom.2016.10.006.
20. Tian Y, Sette A, Weiskopf D. 2016. Cytotoxic CD4 T cells: differentiation, function, and application to dengue virus infection. Front Immunol 7:531. https://doi.org/10.3389/fimmu.2016.00531.
21. Weiskopf D, Sette A. 2014. T-cell immunity to infection with dengue virus in humans. Front Immunol 5:93. https://doi.org/10.3389/fimmu.2014.00093.
22. Grifoni A, Pham J, Sidney J, O'Rourke PH, Paul S, Peters B, Martini SR, de Silva AD, Ricciardi MJ, Magnani DM, Silveira CGT, Maestri A, Costa PR, de-Oliveira-Pinto LM, de Azeredo EL, Damasco PV, Phillips E, Mallal S, de Silva AM, Collins M, Durbin A, Diehl SA, Cerpas C, Balmaseda A, Kuan G, Coloma J, Harris E, Crowe JE, Jr, Stone M, Norris PJ, Busch M, Vivanco-Cid H, Cox J, Graham BS, Ledgerwood JE, Turtle L, Solomon T, Kallas EG, Watkins DI, Weiskopf D, Sette A. 2017. Prior dengue virus exposure shapes T cell immunity to Zika virus in humans. J Virol 91: e01469-17. https://doi.org/10.1128/JVI.01469-17.
23. Reynolds CJ, Suleyman OM, Ortega-Prieto AM, Skelton JK, Bonnesoeur P, Blohm A, Carregaro V, Silva JS, James EA, Maillere B, Dorner M, Boyton RJ, Altmann DM. 2018. T cell immunity to Zika virus targets immunodominant epitopes that show cross-reactivity with other flaviviruses. Sci Rep 8:672. https://doi.org/10.1038/s41598-017-18781-1.
24. Li G, Teleki C, Wang T. 2018. Memory T cells in flavivirus vaccination. Vaccines (Basel) 6:E73. https://doi.org/10.3390/vaccines6040073.
25. Gubler DJ, Halstead SB. 2019. Is Dengvaxia a useful vaccine for

dengue endemic areas? BMJ 367:l5710. https://doi.org/10.1136/bmj.l5710.
26. Thomas SJ, Yoon IK. 2019. A review of Dengvaxia(R): development to deployment. Hum Vaccin Immunother 15:2295–2314. https://doi.org/10.1080/21645515.2019.1658503.
27. Grifoni A, Angelo MA, Lopez B, O'Rourke PH, Sidney J, Cerpas C, Balmaseda A, Silveira CGT, Maestri A, Costa PR, Durbin AP, Diehl SA, Phillips E, Mallal S, De Silva AD, Nchinda G, Nkenfou C, Collins MH, de Silva AM, Lim MQ, Macary PA, Tatullo F, Solomon T, Satchidanandam V, Desai A, Ravi V, Coloma J, Turtle L, Rivino L, Kallas EG, Peters B, Harris E, Sette A, Weiskopf D. 2017. Global assessment of dengue virus-specific CD4(+) T cell responses in dengue-endemic areas. Front Immunol 8:1309. https://doi.org/10.3389/fimmu.2017.01309.
28. Weiskopf D, Angelo MA, Grifoni A, O'Rourke PH, Sidney J, Paul S, De Silva AD, Phillips E, Mallal S, Premawansa S, Premawansa G, Wijewickrama A, Peters B, Sette A. 2016. HLA-DRB1 alleles are associated with different magnitudes of dengue virus-specific CD4+ T-cell responses. J Infect Dis 214:1117–1124. https://doi.org/10.1093/infdis/jiw309.
29. Weiskopf D, Cerpas C, Angelo MA, Bangs DJ, Sidney J, Paul S, Peters B, Sanches FP, Silvera CGT, Costa PR, Kallas EG, Gresh L, de Silva AD, Balmaseda A, Harris E, Sette A. 2015. Human CD8+ T-cell responses against the 4 dengue virus serotypes are associated with distinct patterns of protein targets. J Infect Dis 212:1743–1751. https://doi.org/10.1093/infdis/jiv289.
30. Whitehead SS. 2016. Development of TV003/TV005, a single dose, highly immunogenic live attenuated dengue vaccine; what makes this vaccine different from the Sanofi-Pasteur CYD vaccine? Expert Rev Vaccines 15:509–517. https://doi.org/10.1586/14760584.2016.1115727.
31. Osorio JE, Wallace D, Stinchcomb DT. 2016. A recombinant, chimeric tetravalent dengue vaccine candidate based on a dengue virus serotype 2 backbone. Expert Rev Vaccines 15:497–508. https://doi.org/10.1586/14760584.2016.1128328.
32. Weiskopf D, Angelo MA, Bangs DJ, Sidney J, Paul S, Peters B, de Silva AD, Lindow JC, Diehl SA, Whitehead S, Durbin A, Kirkpatrick B, Sette A. 2015. The human CD8+ T cell responses induced by a live attenuated tetravalent dengue vaccine are directed against highly conserved epitopes. J Virol 89:120–128. https://doi.org/10.1128/JVI.02129-14.
33. Waickman AT, Friberg H, Gargulak M, Kong A, Polhemus M, Endy T, Thomas SJ, Jarman RG, Currier JR. 2019. Assessing the diversity and stability of cellular immunity generated in response to the candidate live-attenuated dengue virus vaccine TAK-003. Front Immunol 10:1778. https://doi.org/10.3389/fimmu.2019.01778.
34. Guy B, Saville M, Lang J. 2010. Development of Sanofi Pasteur tetravalent dengue vaccine. Human Vaccines 6:696–705. https://doi.org/10.4161/hv.6.9.12739.
35. Xu X, Vaughan K, Weiskopf D, Grifoni A, Diamond MS, Sette A, Peters B. 2016. Identifying candidate targets of immune responses in Zika virus based on homology to epitopes in other flavivirus species. PLoS Curr 8:ecurrents.outbreaks.9aa2e1fb61b0f632f58a098773008c4b. https://doi.org/10.1371/currents.outbreaks.9aa2e1fb61b0f632f58a098773008c4b.
36. Kirkpatrick BD, Durbin AP, Pierce KK, Carmolli MP, Tibery CM, Grier PL, Hynes N, Diehl SA, Elwood D, Jarvis AP, Sabundayo BP, Lyon CE, Larsson CJ, Jo M, Lovchik JM, Luke CJ, Walsh MC, Fraser EA, Subbarao K, Whitehead SS. 2015. Robust and balanced immune responses to all 4 dengue virus serotypes following administration of a single dose of a live attenuated tetravalent dengue vaccine to healthy, flavivirus-naive adults. J Infect Dis 212:702–710. https://doi.org/10.1093/infdis/jiv082.
37. Guy B, Barrere B, Malinowski C, Saville M, Teyssou R, Lang J. 2011. From research to phase III: preclinical, industrial and clinical development of the Sanofi Pasteur tetravalent dengue vaccine. Vaccine 29:7229–7241. https://doi.org/10.1016/j.vaccine.2011.06.094.
38. Heinz FX, Stiasny K. 2017. The antigenic structure of Zika virus and its relation to other flaviviruses: implications for infection and immunoprophylaxis. Microbiol Mol Biol Rev 81:e00055-16. https://doi.org/10.1128/MMBR.00055-16.
39. Henry C, Palm AE, Krammer F, Wilson PC. 2018. From original antigenic sin to the universal influenza virus vaccine. Trends Immunol 39:70–79. https://doi.org/10.1016/j.it.2017.08.003.
40. Mongkolsapaya J, Duangchinda T, Dejnirattisai W, Vasanawathana S, Avirutnan P, Jairungsri A, Khemnu N, Tangthawornchaikul N, Chotiyarnwong P, Sae-Jang K, Koch M, Jones Y, McMichael A, Xu X, Malasit P, Screaton G. 2006. T cell responses in dengue hemorrhagic fever: are cross-reactive T cells suboptimal? J Immunol 176:3821–3829. https://doi.org/10.4049/jimmunol.176.6.3821.
41. Rothman AL. 2011. Immunity to dengue virus: a tale of original antigenic sin and tropical cytokine storms. Nat Rev Immunol 11:532–543. https://doi.org/10.1038/nri3014.
42. Weiskopf D, Angelo MA, Sidney J, Peters B, Shresta S, Sette A. 2014. Immunodominance changes as a function of the infecting dengue virus serotype and primary versus secondary infection. J Virol 88:11383–11394. https://doi.org/10.1128/JVI.01108-14.
43. Weiskopf D, Yauch LE, Angelo MA, John DV, Greenbaum JA, Sidney J, Kolla RV, De Silva AD, de Silva AM, Grey H, Peters B, Shresta S, Sette A. 2011. Insights into HLA-restricted T cell responses in a novel mouse model of dengue virus infection point toward new implications for vaccine design. J Immunol 187:4268–4279. https://doi.org/10.4049/jimmunol.1101970.
44. Wen J, Tang WW, Sheets N, Ellison J, Sette A, Kim K, Shresta S. 2017. Identification of Zika virus epitopes reveals immunodominant and protective roles for dengue virus cross-reactive CD8+ T cells. Nat Microbiol 2:17036. https://doi.org/10.1038/nmicrobiol.2017.36.
45. Evavold BD, Allen PM. 1991. Separation of IL-4 production from Th cell proliferation by an altered T cell receptor ligand. Science 252:1308–1310. https://doi.org/10.1126/science.1833816.
46. Sloan-Lancaster J, Allen PM. 1996. Altered peptide ligand-induced partial T cell activation: molecular mechanisms and role in T cell biology. Annu Rev Immunol 14:1–27. https://doi.org/10.1146/annurev.immunol.14.1.1.
47. Carrasco Pro S, Sidney J, Paul S, Lindestam Arlehamn C, Weiskopf D, Peters B, Sette A. 2015. Automatic generation of validated specific epitope sets. J Immunol Res 2015:763461. https://doi.org/10.1155/2015/763461.
48. Vita R, Mahajan S, Overton JA, Dhanda SK, Martini S, Cantrell JR, Wheeler DK, Sette A, Peters B. 2019. The Immune Epitope Database (IEDB): 2018 update. Nucleic Acids Res 47:D339–D343. https://doi.org/10.1093/nar/gky1006.
49. Paul S, Sidney J, Sette A, Peters B. 2016. TepiTool: a pipeline for computational prediction of T cell epitope candidates. Curr Protoc Immunol 114:18.19.11–18.19.24. https://doi.org/10.1002/cpim.12.
50. Bonaldo MC, Gómez MM, Dos Santos AA, Abreu FVSD, Ferreira-de-Brito A, Miranda RM D, Castro MGD, Lourenço-de-Oliveira R. 2017. Genome analysis of yellow fever virus of the ongoing outbreak in Brazil reveals polymorphisms. Mem Inst Oswaldo Cruz 112:447–451. https://doi.org/10.1590/0074-02760170134.
51. Paul S, Weiskopf D, Angelo MA, Sidney J, Peters B, Sette A. 2013. HLA class I alleles are associated with peptide-binding repertoires of different size, affinity, and immunogenicity. J Immunol 191:5831–5839. https://doi.org/10.4049/jimmunol.1302101.
52. Dhanda SK, Mahajan S, Paul S, Yan Z, Kim H, Jespersen MC, Jurtz V, Andreatta M, Greenbaum JA, Marcatili P, Sette A, Nielsen M, Peters B. 2019. IEDB-AR: immune epitope database-analysis resource in 2019. Nucleic Acids Res 47:W502–W506. https://doi.org/10.1093/nar/gkz452.
53. Dhanda SK, Vaughan K, Schulten V, Grifoni A, Weiskopf D, Sidney J, Peters B, Sette A. 2018. Development of a novel clustering tool for linear peptide sequences. Immunology 155:331–345. https://doi.org/10.1111/imm.12984.
54. Dhanda SK, Vita R, Ha B, Grifoni A, Peters B, Sette A. 2018. ImmunomeBrowser: a tool to aggregate and visualize complex and heterogeneous epitopes in reference protein. Bioinformatics 34:3931–3933. https://doi.org/10.1093/bioinformatics/bty463.
55. Grifoni A, Moore E, Voic H, Sidney J, Phillips E, Jadi R, Mallal S, De Silva AD, De Silva AM, Peters B, Weiskopf D, Sette A. 2019. Characterization of magnitude and antigen specificity of HLA-DP, DQ, and DRB3/4/5 restricted DENV-specific CD4+ T cell responses. Front Immunol 10:1568. https://doi.org/10.3389/fimmu.2019.01568.
56. Durbin AP, Kirkpatrick BD, Pierce KK, Elwood D, Larsson CJ, Lindow JC, Tibery C, Sabundayo BP, Shaffer D, Talaat KR, Hynes NA, Wanionek K, Carmolli MP, Luke CJ, Murphy BR, Subbarao K, Whitehead SS. 2013. A single dose of any of four different live attenuated tetravalent dengue vaccines is safe and immunogenic in flavivirus-naive adults: a randomized, double-blind clinical trial. J Infect Dis 207:957–965. https://doi.org/10.1093/infdis/jis936.
57. Kirkpatrick BD, Whitehead SS, Pierce KK, Tibery CM, Grier PL, Hynes NA, Larsson CJ, Sabundayo BP, Talaat KR, Janiak A, Carmolli MP, Luke CJ, Diehl SA, Durbin AP. 2016. The live attenuated dengue vaccine TV003 elicits complete protection against dengue in a human challenge model. Sci Transl Med 8:330ra336.
58. Whitehead SS, Durbin AP, Pierce KK, Elwood D, McElvany BD, Fraser EA, Carmolli MP, Tibery CM, Hynes NA, Jo M, Lovchik JM, Larsson CJ, Doty

EA, Dickson DM, Luke CJ, Subbarao K, Diehl SA, Kirkpatrick BD. 2017. In a randomized trial, the live attenuated tetravalent dengue vaccine TV003 is well-tolerated and highly immunogenic in subjects with flavivirus exposure prior to vaccination. PLoS Negl Trop Dis 11:e0005584. https://doi.org/10.1371/journal.pntd.0005584.

59. Grifoni A, Costa-Ramos P, Pham J, Tian Y, Rosales SL, Seumois G, Sidney J, de Silva AD, Premkumar L, Collins MH, Stone M, Norris PJ, Romero CME, Durbin A, Ricciardi MJ, Ledgerwood JE, de Silva AM, Busch M, Peters B, Vijayanand P, Harris E, Falconar AK, Kallas E, Weiskopf D, Sette A. 2018. Cutting edge: transcriptional profiling reveals multifunctional and cytotoxic antiviral responses of Zika virus-specific CD8(+) T cells. J Immunol 201:3487–3491. https://doi.org/10.4049/jimmunol.1801090.
60. Tian Y, Babor M, Lane J, Seumois G, Liang S, Goonawardhana NDS, De Silva AD, Phillips EJ, Mallal SA, da Silva Antunes R, Grifoni A, Vijayanand P, Weiskopf D, Peters B, Sette A. 2019. Dengue-specific CD8+ T cell subsets display specialized transcriptomic and TCR profiles. J Clin Invest 130:1727–1741. https://doi.org/10.1172/JCI123726.
61. Sette A, Adorini L, Marubini E, Doria G. 1986. A microcomputer program for probit analysis of interleukin-2 (IL-2) titration data. J Immunol Methods 86:265–277. https://doi.org/10.1016/0022-1759(86)90463-1.